# Sonic Hedgehog–dependent proliferation in a series of patients with colorectal cancer

**Richard Douard, MD,[a,b] Stéphane Moutereau, MD,[b] Pascal Pernet, MD, PhD,[d] Mihelaiti Chimingqi, MD,[b] Yves Allory, MD, PhD,[c] Philippe Manivet, MD, PhD,[e] Marc Conti, MD, PhD,[f] Michel Vaubourdolle, MD, PhD,[d] Paul-Henri Cugnenc, MD,[a]** *and* **Sylvain Loric, MD, PhD,[b,d]** *Paris, Créteil, and Le Kremlin Bicêtre, France*

***Background.*** *The Hedgehog (Hh) gene family is known to regulate development of stem cells. In addition, activation is responsible for the induction of GLI1 proto-oncogene and subsequent cellular proliferation. Sonic Hedgehog (SHh), one of the Hh family members promotes carcinogenesis in airway and pancreatic epithelia, is expressed in colonic stem cells. As differentiated colonic cells arise from constant renewal of Hedgehog-expressing colonic stem cells, SHh could be involved in human colonic carcinogenesis.*

***Methods.*** *Tissue samples of colorectal adenocarcinoma (T) and adjacent normal colon tissue (NT) were sampled from each of 44 consecutive patients with colorectal cancer. Specific transcription of SHh, GLI1, and the GLI1 downstream target FOXM1 were evaluated using semiquantitative reverse transcriptase polymerase chain reaction. Similar in vitro measurements of mRNA of GLI1 and FOXM1 transcription levels after specific induction by SHh-Np were performed in the HT-29 colorectal tumor cell line to confirm the in vivo results.*

***Results.*** *SHh mRNA was overexpressed in colorectal adenocarcinomas in 38 of 44 (86%) patients. Expression of transcription levels of GLI1 and FOXM1 correlated with SHh expression (SHh vs GLI1, $r = 0.77$, $P < .0001$; GLI1 vs FOXM1, $r = 0.68$, $P < .0001$; SHh vs FOXM1, $r = 0.79$, $P < .0001$). SHh overexpression did not appear to correlate with the patient characteristics evaluated. Similarly, when studied in the HT-29 colorectal cell line, exogenous SHh promoted cell proliferation, while inhibition of SHh expression decreased proliferation. Expression of GLI1 and FOXM1 mRNA increased with exogenous exposure to SHh.*

***Conclusions:*** *We demonstrated increased expression of SHh mRNA in human colonic adenocarcinomas and in a colorectal cell line with downstream increased expression of GLI1 and FOXM1 mRNA known to promote cell proliferation. This upregulation within human colorectal adenocarcinoma tissue confirms the potential role of the Hh pathway in colorectal carcinogenesis and suggests a potential therapeutic target of Hh blockade in colorectal cancer. (Surgery 2006;139:665-70.)*

*From the General Surgery Department,[a] APHP Georges Pompidou University Hospital, Paris; Clinical Biochemistry-Genetics Laboratory & INSERM EMI 0337[b] and Pathology Department,[c] APHP Henri Mondor University Hospital, Créteil; INSERM U538 & Clinical Biochemistry A Laboratory,[d] APHP Saint-Antoine University Hospital, Paris; Clinical Biochemistry and Molecular Biology Laboratory,[e] APHP Lariboisière University Hospital, Paris; and Clinical Biochemistry Laboratory,[f] APHP Bicêtre University Hospital, Le Kremlin Bicêtre*

Supported by grants from BioQuanta Corp. (Aurora, Colo).

Accepted for publication October 21, 2005.

Reprint requests: Richard Douard, Service de Chirurgie Générale Digestive et Oncologique, Hôpital Européen Georges Pompidou, 20-40 rue Leblanc, 75908 Paris Cedex 15, France. (E-mail: richard.douard@hop.egp.aphp.fr)

0039-6060/$ - see front matter

© 2006 Mosby, Inc. All rights reserved.

doi:10.1016/j.surg.2005.10.012

THE MAJORITY OF ADULT NEOPLASMS arise in tissues such as skin and intestine where somatic stem cells persist to allow constant renewal and maintain tissue integrity. This renewal must be maintained beyond embryogenesis. In neoplastic cells, these mechanisms are overridden to generate a state of uncontrolled proliferation.

Members of the Hedgehog (*Hh*) gene family encode a class of secreted proteins well conserved from invertebrates to mammals that act as an intercellular signals and regulate a large variety of em-

bryonic developmental events; in addition, this gene family is involved in the maintenance of many adult tissues and organs, including skin, lung, brain, bone, and blood.[1,2] At the cell surface, *Hh* signaling activates a receptor complex formed by both patched (PTCH) and smoothened (SMO) transmembrane proteins. By inhibiting SMO in the absence of *Hh* ligands, PTCH silences the *Hh* pathway. Inhibition of PTCH by *Hh* then activates the multifunctional GLI transcription factors. SHh-dependent GLI synthesis induces downstream activation of *Hh* targets such as FOXM1 protein synthesis.[3] The GLI protein subclass of Kruppel-like zinc finger proteins consists of 3 members, among which GLI1 has been shown to function as a protooncogene. Aberrant expression and amplification of *GLI1* is associated with basal cell carcinomas, sarcomas, medulloblastomas, and glioblastomas.[3] FOXM1 belongs to the large family of Winged-helix/Forkhead Box (FOX) transcription factors that play an important role in regulating expression of genes involved in tissue proliferation.[4] FOXM1 may play a role in cell proliferation because its overexpression is associated with accelerated entry into S-phase and mitosis.[3]

Colonic stem cells, which reside near the bottom of the crypts of the colon, appear to divide continually in an asymmetric fashion. The asymmetric division of a stem cell gives rise to one daughter cell that retains the self-renewal properties of her parental stem cell and to a second daughter cell committed to differentiation and eventual cell death, which will either differentiate (into Goblet or Paneth cells) or migrate toward the intestinal lumen.[5] However, if as a result of somatic alterations, normal asymmetric division is dysregulated, stem cells may generate 2 daughter cells with stem cell features that may accumulate and form a neoplasm such as polyp.[6] Among the 3 members of the mammalian hedgehog family, only Sonic hedgehog (SHh) and Indian hedgehog are coexpressed along the gastrointestinal tract in stomach epithelium, at the base of the villi in the small intestine, and in the crypts of the colon.[7,8] Nevertheless, studies on SHh dysregulation in human colorectal cancer and its paired colorectal normal tissue have not been reported before.

Prompted by the evidence of the ability of Hh signaling to promote tumor growth[9] as well as the recently reported implication of SHh in regeneration and carcinogenesis of the airway epithelium,[10] we investigated the potential role of SHh in human colonic carcinogenesis in vivo using adenocarcinomas from colorectal cancer patients and in vitro using the colorectal HT-29 cell line.

**Table.** Clinical features of the 44 colorectal cancer patients

| *Patients* | *Population* |
|---|---|
| N | 44 |
| Age (mean ± SD) | 69 ± 13 |
| Tumor location* | |
| Right | 12 |
| Left | 4 |
| Sigmoid | 12 |
| Rectum | 16 |
| UICC tumor staging | |
| Stage II | 20 |
| Stage III | 12 |
| Stage IV | 12 |
| Tumor size (cm, mean ± SD) | 4.1 ± 2.0 |

*SD,* Standard deviation; *UICC,* International Union Against Cancer.
*Primary neoplasms that developed within the sigmoidorectal junction and the left third of the transverse colon were considered rectal and left colon cancers, respectively.

## PATIENTS AND METHODS

The study population consisted of 44 consecutive patients with colorectal cancer admitted for elective colorectal operations (Table). Preoperative evaluation included a chest radiograph, abdominal ultrasonography, and colonoscopy. All cancer patients were operated on and the primary neoplasm resected. Adenocarcinomas were staged according to pTNM/International Union Against Cancer (UICC) classification. None of the patients received neoadjuvant chemotherapy before operation. After surgical resection, tissue samples of colon tumor (T) and adjacent normal colon tissue (NT) were sampled from each patient and frozen immediately in liquid nitrogen. None of the patients had an identified genetic predisposition for colon cancer such as familial adenomatous polyposis or hereditary nonpolyposis colorectal cancer.

To further confirm clinical data, the HT-29 colorectal tumor cell line was evaluated because HT-29 cells display autocrine Hh signaling and express PTCH and SMO Hh receptors and have fully functional downstream Hh signaling.[8-14] Two different conditions were tested on cell lines: (1) exogenous SHh effect was evaluated using exogenous recombinant SHh aminoterminal peptide SHh-Np alone (R&D systems Europe, Lille, France) or in combination with a goat polyclonal anti-SHh antibody (N19, Santa Cruz Biotechnology, Santa Cruz, Calif), (2) the effect of cyclopamine, an inhibitor of cholesterol synthesis known to inhibit correct SHh signaling[11] (Toronto Research Chemicals Inc, Toronto, Canada). After culturing HT-29 cells for 24 hours to 72 hours in 1% fetal calf serum in the

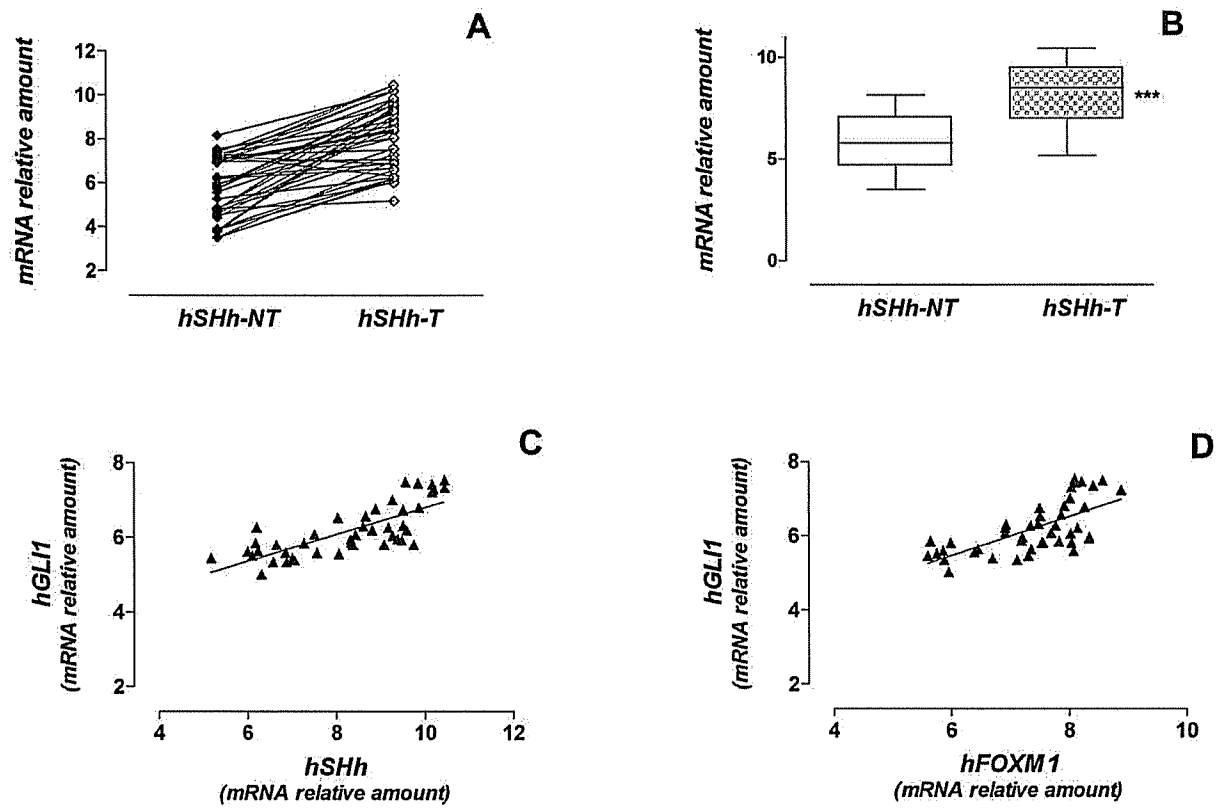


**Fig 1.** Hh pathway expression in patient tissues. **A**, Paired quantification of neoplastic (*SHh* T) and non-neoplastic (*SHh* NT) *SHh* transcripts. **B**, Median values of patient neoplastic (*SHh* T) and non-neoplastic (*SHh* NT) *SHh* transcript (***$P < .0001$). **C**, Neoplasm *SHh* transcripts and downstream *GLI1* mRNA production correlate. **D**, GLI activation correlates with downstream *FOXM1* production.

presence or absence of SHh pathway activators/inhibitors, of the SHh pathway cell proliferation was measured by determining in triplicate the viable cell mass with optical density measurements at 490 nM using CellTiter96 colorimetric assay (Promega France, Charbonnière, France).

To ensure that colonic tissues really contained neoplastic cells, frozen tissues were stained initially with H&E and further examined by a pathologist. Then, only samples that had more than 80% of neoplastic tissue were selected in the so-called T group. Thus, total RNA was extracted from 30-$\mu$m sections from non-neoplastic and neoplastic tissues, and extracts from HT-29 cells via the acid-phenol-guanidinium method and reverse transcribed using Superscript II reverse transcriptase and random hexamers as templates. Specific transcripts for *SHh*, *GLI1*, and *FOXM1* were quantified using semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) on a PerkinElmer (Wellesley, Mass) ABI7700 apparatus. Specific primers designed with PC-RARE software (Eurogentec, Seraing, Belgium) were used (GLI1-F, ttccaatgagaagccgtatg; GLI1-R, gggacacagtggtaccggtg; FOXM1-F, acatccagtggcttcgaaag; FOXM1-R, ggcggagctctggattcggt; SHh-F, cagaaactccgagcgattta; SHh-R, actgcgcggccctcgtagtg), and real-time amplification using standard protocols (2 steps: 30" 94°C denaturation, 30" annealing/extension at 60°C) was followed using SYBR-Green fluorescence emission. This process was validated initially by sequencing purified amplimers and then blasting against annotated public databases. Each value corresponds to a relative expression level for the corresponding transcripts designed, according to Bieche et al [12] as follows: (1) Each sample was normalized on the basis of the expression of the housekeeping gene cyclophilin A[13]; (2) the relative level of target gene expression was then normalized to a calibrator consisting of a normal colon tissue sample that contained the smallest accurately quantifiable mRNA amount of interest. The calibrator allowed determination of the limit of assay quantification of each target, which corresponds to a Ct value of 38 for *SHh*, *GLI1*, and *FOXM1*. The normalized value of each target for each sample was divided by the calibrator normalized value to give the final relative ex-

pression level (Fig 1). Consequently, each value corresponds to a relative expression level for the corresponding transcripts

Quantitative variables were compared using Student's *t* test, and correlation tests were made on GraphPad Prism software (GraphPad Software, San Diego, Calif). Results are expressed as mean ± SD. Statistical significance was $P < .05$.

## RESULTS

In 38 of 44 patients (86%), *SHh* expression in T was increased compared with the NT. In the 6 remaining patients, this level was comparable in T versus NT colon tissue samples (Fig 1, *A*). A large increase in *SHh* transcripts within neoplastic tissue was demonstrated when comparing the T versus NT tissues (Fig 1, *A* and *B*; $P < .0001$). In parallel, *GLI1* expression was greater in tumor tissue than in its normal counterpart (6.18 ± 0.68 vs 3.61 ± 1.24, respectively, $P < .0001$). Within neoplastic tissue, *GLI1* mRNA expression correlated quantitatively with expression of *SHh* transcripts (Fig 1, *C*; r = 0.77, $P < .0001$). The level of expression of *SHh*, *GLI1*, and *FOXM1* transcripts were not associated with either tumor location, pTNM/UICC stage, tumor differentiation, tumor size, or tumor extension. Because *GLI1* activation seems to be highly dependent on *SHh*, we tested for downstream activation of *FOXM1* production; *FOXM1* was found to be overexpressed in T versus NT sample tissues (7.37 ± 0.88 vs 3.93 ± 1.16; $P < .0001$). A strong correlation was found between expression of *GLI1* and *FOXM1* (Fig 1, *D*; r = 0.68, $P < .0001$) and between expression of *SHh* and *FOXM1* (r = 0.79, $P < .0001$). After normalization using the normal tissue counterpart, the same correlations were observed (*SHh* vs *GLI1*, r = 0.66, $P < .0001$; *GLI1* vs *FOXM1*, r = 0.58, $P < .005$).

To confirm these in vivo data, we performed additional in vitro experiments on the colon adenocarcinoma-derived HT-29 cells that display autocrine Hh signaling (HT-29 cells). We analyzed the effect of either endogenous autocrine SHh production or exogenous SHh-Np on HT-29 cell proliferation. As shown in Fig 2, *A*, cyclopamine decreased HT-29 cell proliferation compared with untreated controls (about 30% reduction 72 hours after exposure). Likewise, exogenous SHh-Np increased HT-29 cell proliferation (by 2-fold after 72-hour exposure to 0.50 μg/mL SHh-Np). This pro-proliferative effect was abrogated almost completely when a specific anti-SHh antibody was added concomitantly at day 0 to the culture medium. Expression of Hh-dependent downstream transcription factors confirmed that HT-29 cells display both fully functional Hh receptors and a transduction system because SHh-Np induced *GLI1* and *FOXM1* transcription in a nonlinear, dose-dependent manner (Fig 2, *B*). Similar results were found with CaCo2 colon cancer cell line (data not shown).

## DISCUSSION

Because normal stem cells and cancer cells share the ability to self-renew, it seems reasonable to propose that newly arising cancer cells may appropriate the machinery for self-renewing cell division expressed normally in stem cells. Some signaling pathways, such as the Notch, Wnt, and Hh signaling pathways, that regulate stem cell renewal may also be involved in carcinogenesis. [10] Colon cancer may represent a malignancy arising from an epithelium progenitor cell that retains primitive features of differentiation that undergoes regulation via Hh signaling.[5,7]

Our results show a strong increase of *SHh* gene transcription in neoplastic tissue in patients with colorectal adenocarcinoma compared with normal tissue from each patient in 86% of patients. Within neoplastic tissue of colorectal cancer patients, *SHh* activation was associated with downstream activation of *GLI1* and *FOXM1* transcription factors known to promote cell proliferation through induction of the cyclin genes induction.[9,15] We also showed that blocking endogenous synthesis of *SHh* decreased HT-29 cell proliferation, whereas activation by the recombinant SHh-Np peptide induced *GLI1* and *FOXM1* expression and subsequent cell proliferation. These data provide evidence that not only the ligand SHh, but also its downstream targets (*GLI* and *FOXM1*), are overexpressed in human colorectal cancer tissue and confirm very recent data that have shown that cell regulation by Hh signaling is altered in colonic neoplasia.[14,16,17] Berman et al[17] were the first to provide evidence for involvement of the Hh pathway in regulating normal or neoplastic tissues in the digestive tract. These investigators suggested that Hh functions as an autocrine inducer of cell proliferation. Nevertheless, despite a strong expression of *SHh* and *IHh* in colonic tissues, the absence of *PTCH*, a variable *GLI1* transcript expression, and a moderate effect of cyclopamine in a subset of colon cell lines have challenged the influence of the Hh pathway on colonic cell proliferation. More recently, Qualtrough et al[14] showed that cyclopamine, which inhibits Hh signaling, induces apoptosis in human colonic cell cultures, and Oniscu et al[16] showed a dysregulation of the *SHh* pathway in archived, nonpaired human tissue slides (neoplastic, adenomas, non-neoplastic tissue). These studies provide evi-

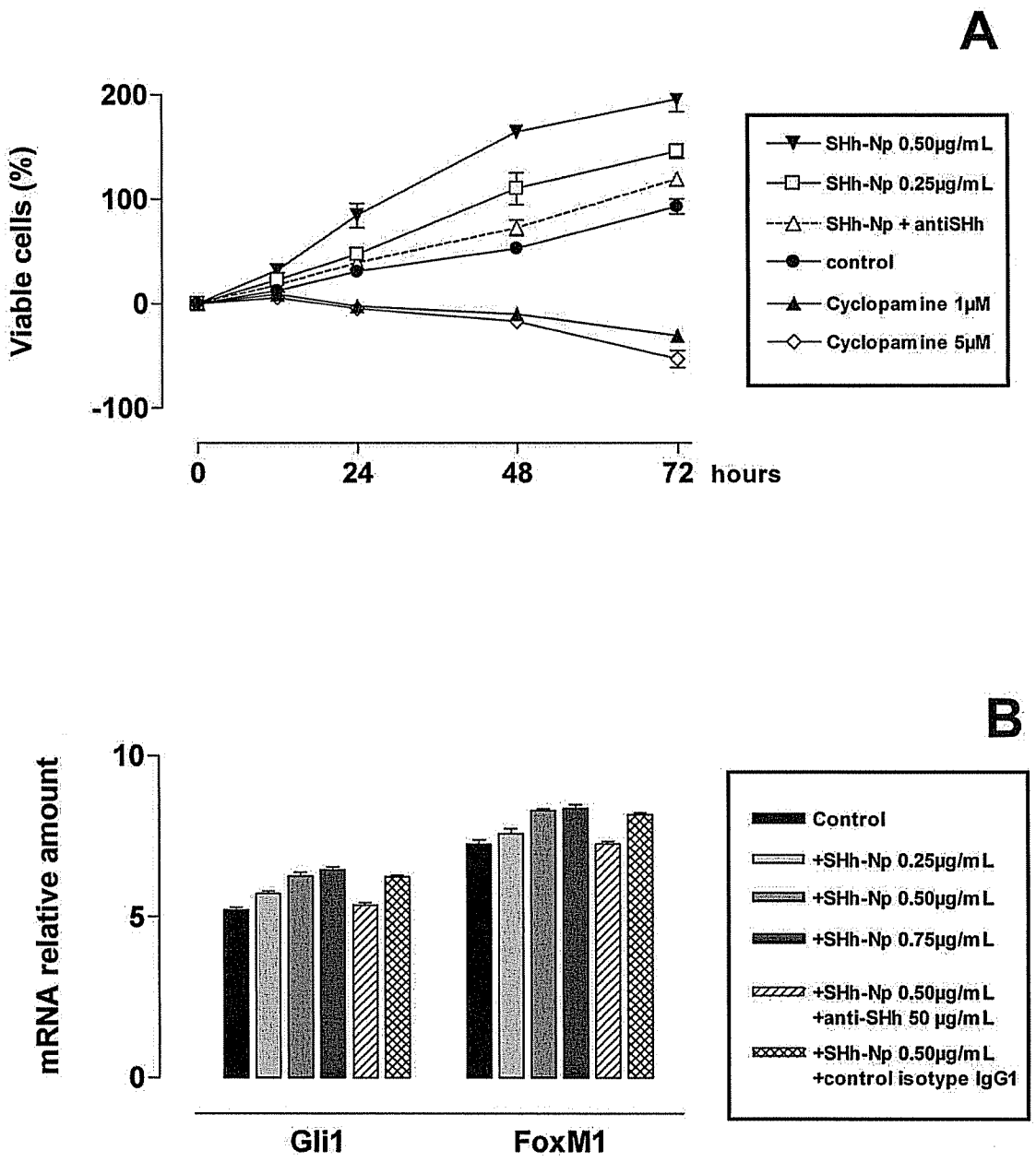


**Fig 2.** Effects of altering the Hh pathway in vitro in HT-29 cells. **A**, Effect of exogenous SHh-Np without or with anti-SHh antibody and exogenous cyclopamine on cell proliferation. **B**, Effect of exogenous SHh-Np on expression of GLi-1 and FOXM1.

dence for the role of the Hh pathway in normal and neoplastic colonic tissue proliferation. Compared with these recent publications, our study provides confirmative evidence for *SHh* dysregulation in a prospective human study of neoplastic and adjacent non-neoplastic tissues paired for each patient.

The complex network of mechanisms governing the specific regulation of the Hh signaling cascade remains to be fully elucidated. Recently, SHh has been shown to be an important promoter of normal colonic cell growth. Nevertheless, SHh overexpression suggests that the Hh signaling pathway may play a central role in the pathogenesis of colon cancer and that maintenance of Hh signaling is important for colon cancer tumorigenesis. In addition, SHh activation does not seem to be correlated with either pTNM/UICC stage, location of the neo-

plasm in the colon, or tumor size, which might support the concept that SHh dysregulation is an early event in colon cancer carcinogenesis already present in colonic polyps.[6]

To the best of our knowledge, our study is the first assessing comparative SHh expression and signaling in both normal and neoplastic tissue of human patients with colorectal adenocarcinoma, confirming the role of SHh suspected previously in animal and in vitro studies. A major challenge for the future will be to modulate Hh signaling as a potentially effective treatment for patients with colorectal adenocarcinoma containing Hh-induced aberrant signaling. Therefore, Hh blockade, may represent a new therapeutic approach to colorectal cancer in humans as promising as the one recently reported in mice.[17]

The authors thank Pierre Ratier for help in preparation of this report and constant support.

**References**

1. Mullor JL, Sanchez P, Altaba AR. Pathways and consequences: Hedgehog signaling in human disease. Trends Cell Biol 2002;12:562-9.
2. Lum L, Beachy PA. The Hedgehog response network: sensors, switches, and routers. Science 2004;304:1755-9.
3. Teh MT, Wong ST, Neill GW, et al. FOXM1 is a downstream target of Gli1 in basal cell carcinomas. Cancer Res 2002;62: 4773-80.
4. Lehmann OJ, Sowden JC, Carlsson P, et al. Fox's in development and disease. Trends Genet 2003;19:339-44.
5. de Santa Barbara P, van den Brink GR, Roberts DJ. Development and differentiation of the intestinal epithelium. Cell Mol Life Sci 2003;60:1322-32.
6. Luebeck EG, Moolgavkar SH. Multistage carcinogenesis and the incidence of colorectal cancer. Proc Natl Acad Sci U S A 2002;99:15095-100.
7. Ramalho-Santos M, Melton DA, McMahon AP. Hedgehog signals regulate multiple aspects of gastrointestinal development. Development 2000;127:2763-72.
8. van den Brink GR, Bleuming SA, Hardwick JC, et al. Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation. Nat Genet 2004;36:277-82.
9. Duman-Scheel M, Weng L, Xin S, Du W. Hedgehog regulates cell growth and proliferation by inducing Cyclin D and Cyclin E. Nature 2002;417:299-304.
10. Taipale J, Beachy PA. The Hedgehog and Wnt signalling pathways in cancer. Nature 2001;411:349-54.
11. Cooper MK, Wassif CA, Krakowiak PA, et al. A defective response to Hedgehog signaling in disorders of cholesterol biosynthesis. Nat Genet 2003;33:508-13.
12. Bieche I, Nogues C, Paradis V, et al. Quantitation of hTERT gene expression in sporadic breast tumors with a real-time reverse transcription-polymerase chain reaction assay. Clin Cancer Res 2000;6:452-9.
13. de Kok JB, Roelofs RW, Giesendorf BA, et al. Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. Lab Invest 2005;85: 154-9.
14. Qualtrough D, Buda A, Gaffield W, et al. Hedgehog signalling in colorectal tumour cells: induction of apoptosis with cyclopamine treatment. Int J Cancer 2004;110:831-7.
15. Wang X, Kiyokawa H, Dennewitz MB, Costa RH. The Forkhead Box m1b transcription factor is essential for hepatocyte DNA replication and mitosis during mouse liver regeneration. Proc Natl Acad Sci U S A 2002;99:16881-6.
16. Oniscu A, James RM, Morris RG, et al. Expression of Sonic hedgehog pathway genes is altered in colonic neoplasia. J Pathol 2004;203:909-17.
17. Berman DM, Karhadkar SS, Maitra A, et al. Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. Nature 2003;425:846-51.